Ablation of Gene Expression of N-Methyl-D-Aspartate Receptor One by Antisense Oligonucleotides in Striatal Neurons in Culture

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Key Words

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Abstract

In the present study, a twenty-mer antisense oligonucleotide specific for N-methyl-D-aspartate receptor one (ANR1) was applied to striatal neurons in primary cell culture. The ANR1 was found to be specific and nontoxic. Significant reductions in expression of NR1 mRNA and proteins were resulted after a single dose of ANR1 transcripts. Interestingly, there were reductions in total NR1 proteins but two phosphorylated forms of NR1 proteins at serine 896 and 897 residues were not reduced. There was also no change in the pattern of distribution of NR1 immunoreactivity in the striatal neurons. In addition, significant reductions of NMDA-mediated peak inward current were found after application of a higher concentration of ANR1 (20–100 μ M) by patch clamp recordings. The present results indicate that ANR1 is a use-

ful agent in reducing NMDA receptor functions. The present data thus provide detailed cellular and molecular mechanisms to explain our previous findings of amelioration of motor symptoms in a rat model of Parkinson's disease. More importantly, application of ANR1 was also found to display neuroprotective effects of striatal neurons against NMDA-induced excitotoxic cell death. The findings have implications in development of new approach in prevention of cell death in neurodegenerative diseases and new treatments for these diseases.

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Introduction

Glutamate-induced excitotoxic cell death is implicated in neurodegenerative diseases [1, 2], including motor disorders, namely Parkinson's disease [3–5] and Huntington's disease [6, 7]. N-methyl-D-aspartate (NMDA) receptors are one of the major classes of ionotropic glutamate receptors that mediate excitotoxic cell death [8, 9].

Functional native NMDA receptors are composed of heteromeric subunits (NR1, NR2A-D and NR3A-B) [10–20] and their functions depend upon subunit composi-

tions [10–15]. Previous findings indicate that NR1 is the major subunit that forms a functional NMDA channel. The roles of NR2 subunits are thought to modulate the functions of the ion channel.

Degeneration of neurons in the basal ganglia is a hallmark for motor disorders [21]. A degeneration of medium spiny neurons, the striatal projection neurons, in the neostriatum is the cause of Huntington's disease [22]. In the neostriatum, NMDA receptors are found to be expressed in most of the subpopulations of striatal neurons including the medium spiny neurons [23] and involved in motor regulation in the neostriatum and circuitry in the basal ganglia [24, 25].

Recent findings in our laboratory [26–28] and others [29] indicate that intrastriatal administrations of antisense transcripts specific for NMDA receptor subunits, i.e. antisense specific for NR1 subunit (ANR1) and NR2B subunit, into rats results in reductions of NR1 and NR2B mRNA and protein expressions. Interestingly, intrastriatal administrations of ANR1 in 6-hydroxydopamine-lesioned rats, an animal model of Parkinson's disease, induce an amelioration of motor symptoms in the rats [28]. ANR1 is therefore thought to be a potential antiparkinsonian agent and may have applications in treating other related motor disorders such as Huntington's disease, which is primarily resulted from a degeneration of striatal neurons [7].

Despite our previous observations, physiological and pharmacological effects of ANR1 to striatal neurons is, however, not clear. The objectives of the present study were to elucidate the cellular and molecular effects of ANR1 in striatal neurons. Striatal neurons *in vitro* were employed. Importantly, the efficacy of ANR1 in prevention of excitotoxic cell death was assessed.

Preliminary data of the present study were reported in abstract form [30, 31].

Experimental Procedures

Primary Cell Culture of Striatal Neurons

In the present study, neonatal rats (postnatal day 1, Sprague-Dawley) were used. The handling of rats and all procedures that performed on them were approved in accordance with the Animals (Control of Experiments) Ordinance, Hong Kong, PR China, by the Committee on the Use of Human and Animal Subjects in Teaching and Research, Hong Kong Baptist University, and conformed to the *Principles of Laboratory Animal Care* [NIH publ. No. 86-23, revised 1985]. The rats were sacrificed by decapitation and brain region of the neostriatum was dissected in a dissecting microscope [32]. The brain tissues were chopped using a razor blade in minimal essential medium (Gibco, BRL). The chopped tissues were

then incubated in trypsin/EDTA solution with 1% penicillin-streptomycin (Gibco) and B27 supplement (Gibco) for 1 h at 37°C [32]. Agitation of the medium was performed every 15 min. Single-cell suspension was obtained by dissociation of the tissues in Neurobasal medium with soybean trypsin inhibitor (Gibco) and DNase 1 (Boehringer Mannheim) for 30 min at 37°C. Cell pellets were obtained by centrifugation (1,200 rpm for 5 min). The pellets were washed and re-suspended in culture medium (Neurobasal medium, 1% penicillin-streptomycin, B27 supplement; Gibco; and 10% fetal bovine serum; Sigma). The neurons were then plated on poly-D-lysine-coated 6-well plastic plates or on glass coverslips. The cultures were maintained at 37°C in CO₂ incubator (5% CO₂). Cytosine arabinoside (10 µM, Fluka) was added to the culture 2 days after plating in order to inhibit the growth of glial cells [33]. Treatments on the cultures were conducted at 6 days *in vitro* [32].

Oligonucleotide Treatments

Oligonucleotide transcripts were designed on the basis of the known sequences of rat NR1 [26, 29]. All transcripts used in the present study contained 20 phosphorothiolated nucleotides and were purchased commercially (Gibco). ANR1 transcripts were proven by our laboratory to enable specific knockdown of NR1 protein expression in rat *in vivo* [26, 34]. The sequences of the oligos were as follows: 5'-G CAG GTG CAT GGT GCT CAT G-3' (ANR1), 5'-C ATG AGC ACC ATG CAC CTG C-3' (NR1 sense: SNR1), 5'-A ACG TGA GCG TGG TCC TAG T-3' (randomized sequence based on the NR1 sequence: RNR1). ANR1, SNR1 and RNR1 were dissolved in 0.9% saline at a concentration of 2.5 mM and stored at –20°C prior to use. A single dose of 5, 20 or 100 µM oligos was added to the cultured striatal neurons at 6 days *in vitro*. The cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere. Twenty-four hours after the treatment, the cells were harvested for reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. In addition, 30 h after the treatment, the cells were harvested for Western blot analysis, NMDA toxicity assay, or whole-cell patch clamp analysis.

Reverse Transcriptase-Polymerase Chain Reaction

All glass- and plastic-ware were treated with diethyl pyrocarbonate and autoclaved. TRIZOL Reagent (Gibco) was added to striatal neurons at 24 h after the oligo treatments. After 5 min incubation at room temperature, chloroform was added for phase separation. The upper aqueous phase was collected and the RNA was precipitated by mixing with isopropyl alcohol. The RNA pellet was washed once with 75% ethanol and was air-dried. It was finally re-dissolved in RNase-free water. A₂₆₀/A₂₈₀ ratios were between 1.6 and 1.8.

Semiquantitative RT-PCR was conducted as described previously using the housekeeping gene GAPDH as an internal standard [26, 27, 35, 36]. RT-PCR was performed using Ready-To-Go RT-PCR beads (Amersham Pharmacia Biotech) and was calibrated using GAPDH and NR1, NR2A, NR2B, NR2C or NR2D primer pairs (see below). Different amplification cycles were used to allow detection of the amplified products while keeping amplification for these genes in the log phase (NR1, 33 cycles; NR2A, 47 cycles; NR2B, 31 cycles; NR2C, 48 cycles; NR2D, 43 cycles; GAPDH, 26 cycles; results of the NR1 control experiment are shown in fig. 1).

Total RNA was diluted to 1 µg/µl in RNase-free water, mixed with 0.5 µg of pd(N)₆ and 47 µl of RNase-free water to a final volume of 49 µl in a reaction tube containing RT-PCR beads. The reaction was incubated at 42°C for 30 min, followed by 95°C for

5 min to inactivate the RT and to completely denature the template. Gene-specific primer sets were constructed as previously described [26, 27, 37, 38] (NR1: 5'-AACCTGCAGAACCGCAAG-3' and 5'-GCTTGATGAGCAGGTCTATGC-3'; NR2A: 5'-TCCATTC-TTCTGTCATCCTGC-3' and 5'-AAGACCGTCTCTCACTCTT-GC-3'; NR2B: 5'-TGCACAATTACTCCTCGACG-3' and 5'-TCCGATTCTTCTTCTGAGCC-3'; NR2C: 5'-TTGAGGACAA-CGTGGACACC-3' and 5'-TCCAGTCGTATTCTCCAGC-3'; NR2D: 5'-GCACTTGCATCAGAGACTCG-3' and 5'-CTCAC-CAATCATGCCATTCC-3'). One of the primer sets was added to give a final volume of 50 μ l. Reactions were run for the optimal cycles with 55°C annealing cycle (1 min), 72°C extension cycle (1 min), and a 95°C denaturing cycle (50 s). The GAPDH (5'-AT-GGTGAAGGTCGGTGTGAAC-3' and 5'-GCTGACAATCTT-GAGGGAGT-3') primer set [35] was added into the reaction according to its corresponding pre-calibrated cycle number. Control amplifications were done either without RT or without RNA. Amplification from primers, NR1, NR2A, NR2B, NR2D and GAPDH produces a fragment of 333, 224, 222, 224 and 437 bp in length, respectively [26, 27, 37, 38]. Images of the gels after RT-PCR analyses were digitally captured by a gel documentation system (Ultra Violet Products C-80). Fluorescence intensity of each band was then semiquantified by an image analyzing software (Metamorph, Universal Imaging, Downingtown, Pa., USA). The changes of intensity of NR1 mRNA after the ODN treatments were normalized using the intensity obtained in the GAPDH bands and the ratios were expressed as mean $\pm$ SEM [27, 37]. Data obtained from at least three separate treatments were averaged and compared with other groups by ANOVA with Duncan's multiple range tests.

Western Blot Analysis

Western blot analysis was performed in accordance with a previous study [39]. Thirty hours after the oligo treatments, neurons in culture were washed ($3 \times$ PBS) and lysed in buffer (in mM: sucrose 250, imidazole/HCl 50, sodium EDTA 2 and β -mercaptoethanol, pH 7.2). The lysate was collected and centrifuged at 14,000 g for 15 min. The supernatant was stored at -70°C . The quantity of proteins in the lysate was determined using a commercially available kit (Biorad Protein Assay Kit). Proteins (100 μ g/well) were submitted to SDS-polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes using a commercially available system (Bio-Rad Mini-protein 3 and mini trans-blot electrophoretic transfer for cell). The membranes were subjected to Western blot analysis. Total NR1 proteins were revealed by NR1 specific primary antibodies (rabbit polyclonal anti-NR1, 0.05 μ g/ml in Tris-buffered saline supplied with 2% nonfat skim milk, 0.05 M, pH 7.4 [TBS], Chemicon; or mouse monoclonal anti-NR1, 1 μ g/ml in TBS, Upstate Biotechnology). In addition, two phosphorylated forms of NR1 proteins were also revealed using specific antibodies (rabbit polyclonal anti-phospho-ser896-NR1, 1 μ g/ml in TBS, and rabbit polyclonal anti-phospho-ser897-NR1, 1 μ g/ml in TBS, all from Upstate Biotechnology). In control experiments, proteins from striatal neurons were treated with 500 units of lambda protein phosphatase in phosphatase reaction buffer (from Upstate Biotechnology) for 4 h at 30°C prior to the phosphorylated NR1-specific antibody treatments. The membranes were incubated in primary antibodies for 2 h at room temperature. The membranes were then incubated in appropriate secondary antibodies (horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG, Santa Cruz, 1:5,000 in TBS with 2% non-

Fig. 1. Amplification of NR1 subunit from striatal neurons with different PCR cycles is shown. The optimum cycle is shown here as 33 cycles (indicated by an arrow).

fat skim milk) for 1 h at room temperature. Bands in the membranes were visualized using chemiluminescence detection (ECL Western blotting detection reagents; Amersham Pharmacia Biotech; the above procedure was performed in accordance with the supplier). Images of the bands were developed on films and captured by a computer with a scanner. Optical density of each band was then semiquantified by software (Metamorph) and statistically analyzed by one-way ANOVA with Duncan's multiple range tests.

Double Immunofluorescence

Thirty hours after oligo treatments, striatal neurons were washed ($3 \times$ phosphate-buffered saline [PBS], pH 7.4) prior to immunofluorescence. Immunoreactivity for NR1 plus glutamic acid decarboxylase 67 (GAD; a specific neurochemical marker for GABAergic striatal neurons) was revealed in a single reaction for comparison. Double immunofluorescence was performed as previously described [32]. Briefly, neurons were incubated in a mixture of primary antibodies, namely, NR1 (rabbit polyclonal; 0.5–1.0 μ g/ml in PBS supplemented with 2% NGS and 0.1% Triton X-100 [PBS-Triton]; Chemicon) with GAD (mouse monoclonal; 1:2,000 in PBS-Triton; Chemicon) for 2 h at room temperature. The neurons were then washed ($3 \times$ PBS) and incubated with a mixture of secondary antibodies for 1 h at room temperature. Goat anti-rabbit IgG conjugated with Alexa 488 (1:200 in PBS-Triton, Molecular Probes) and goat anti-mouse IgG conjugated with cy5 (1:200 in PBS-Triton, Chemicon) were used. The neurons were then washed ($3 \times$ PBS) and mounted on glass slides with mounting medium (Vectashield, Vector Labs). The neurons were then observed in a laser scan confocal microscope (Zeiss LSM 510; excitation at 488 and 633 nm). Digital images of the double-labeled neurons showing immunoreactivity for NR1 and GAD were captured under the same parameters in the confocal microscope.

Fig. 2. Co-amplification of NR1, NR2 subunits and GAPDH obtained from striatal neurons treated with NR1 antisense oligos (ANR1), NR1 sense oligos (SNR1), NR1 random oligos (RNR1) and control. **A** A band of NR1 PCR product of 333 bp is observed in each of the four groups. The level of NR1 mRNA is only seen to be reduced after ANR1 treatment when compared to the other three groups (indicated by asterisk). Similar levels of NR1 mRNA are found in the sense NR1-, random NR1-treated striatal neurons and in control striatal neurons. **B–D** The levels of NR2A (224 bp), NR2B (222 bp) and NR2D (224 bp) mRNAs remain the same in all four groups of striatal neurons.

Control for Immunocytochemistry

Control experiments for double immunofluorescence were carried out by omitting either primary or secondary antibody solutions in the above reaction sequence. The cells were then observed in the fluorescence microscope or in the confocal microscope [32].

Whole-Cell Recordings

Whole-cell patch clamp recording experiments were performed in accordance to a previous study [32]. Striatal neurons in primary cell culture were cultured on poly-*D*-lysine-coated coverslips. The coverslips with neurons were transferred to a small-volume recording chamber that was mounted on an upright microscope. The neurons were continuously superfused at 2.5–3 ml/min with artificial cerebrospinal fluid (ACSF) of the following composition (in mM): NaCl 125, KCl 2.0, CaCl₂ 2.5, KH₂PO₄ 1.2, glucose 11 and NaHCO₃ 26. The ACSF was continuously bubbled with 95% O₂ and 5% CO₂ and maintained at 35 ± 1°C. Whole-cell patch-clamp recordings were obtained from striatal neurons at 30 h after ODN treatments. The pipettes typically had a resistance of 3–4 MΩ when filled with an internal solution of the following composition (in

mM): K-gluconate 140, EGTA 1.0, MgCl₂ 2.0, Na₂ATP 2.0, Tris GTP 0.4, HEPES 10, with pH adjusted to 7.25–7.30. Application of NMDA was achieved by switching the control ACSF to one containing a known concentration of the drug. NMDA-induced currents were measured at a holding potential of –60 mV. The peak inward currents were expressed as mean ± SEM and compared statistically by one-way ANOVA with Tukey's post hoc tests. In some experiments, biocytin (1%) was included in the internal solution to label the recorded neuron to facilitate identification during subsequent immunostaining for GAD.

NMDA Toxicity Assay

Thirty hours after the oligo treatments, the striatal neurons were subjected to NMDA toxicity experiments as described previously [33]. Neurons were washed in glutamate-free Locke's solution (in mM: NaCl 128, KCl 5, MgCl₂ 1.2, CaCl₂ 2.7, Na₂HPO₄ 1, HEPES 5 and glucose 10; pH 7.4) for 10 min at 37°C. Neurons were then incubated with 500 μM NMDA, 1,000 μM NMDA, or 500 μM NMDA plus 1 mM DL-2-amino-5-phosphonovaleate (DL-APV, Tocris) in Mg²⁺-free Locke's solution in the presence of 3 μM gly-

Table 1. Statistical analysis of the level of fluorescence of the bands after RT-PCR (ratios between the NR and GAPDH bands)

PCR product	Control	RNR1 treatment	SNR1 treatment	ANR1 treatment
NR1	1.53 ± 0.06	1.51 ± 0.03	1.33 ± 0.07	0.82 ± 0.05*** [−43.16 ± 2.57%]
NR2A	1.07 ± 0.03	1.12 ± 0.02	1.12 ± 0.07	1.08 ± 0.01
NR2B	1.37 ± 0.08	1.34 ± 0.07	1.32 ± 0.07	1.29 ± 0.06
NR2D	1.26 ± 0.05	1.25 ± 0.07	1.27 ± 0.05	1.21 ± 0.05

Means ± SEMs are shown. Statistical analysis was performed using one-way ANOVA with Duncan's multiple range tests. ***p < 0.001.

cine. The neurons were rinsed with Locke's solution and incubated in culture medium for 24 h at 37°C. Control experiments were performed in neurons of the same culture periods using the same treatments of the Mg²⁺-free Locke's solution without NMDA.

After treatments, digital images of neurons were captured by a digital camera in the microscope. Percentage of cell death in cultures after exposure to NMDA and in control experiments was assessed by determination of the enzymatic activity of lactate dehydrogenase (LDH) in the culture medium using a commercially available cytotoxicity detection kit (cytotoxicity detection kit [LDH], Roche Molecular Biochemicals). Protocols were employed in accordance to the supplier's information. The percentage of cell death was estimated by measuring the optical density of the tested samples using a spectrophotometer (iEMS analyzer, Labsystems). The percentage of cell death in those cells treated with NMDA was then normalized by the values of the control experiments. All data are presented as means ± SEM and statistical analyses were performed using one-way ANOVA with Duncan's multiple range tests.

Results

Effects of ANR1 Treatments on Expression of NMDA Receptor mRNAs

Data were obtained from twenty four separate RT-PCR experiments. No detectable level of NR2C mRNA was found in the striatal neurons in culture (data not shown) and this was consistent to our previous report [32]. Six hours after treatments using a single dose of 5 μM of ANR1, RT-PCR showed that there were no changes in the mRNA levels of the NR1, NR2A, 2B and 2D mRNAs when compared to the SNR1-treated, RNR1-treated or controls (data not shown).

Subsequently, 24 h after the ANR1 application, there was a pronounced reduction in the intensity of NR1 mRNA (fig. 2A). Statistical analysis of the PCR products indicated that this was a significant decrease (table 1). Treatments with SNR1 and RNR1 did not affect the NR1 mRNA levels in the striatal neurons (fig. 2A; table 1). Similarly, treatments with 20 and 100 μM of ANR1 re-

sults in similar degree of reduction of NR1 mRNA levels (data not shown).

In addition, same as the 6 h treatments, no modulation in the levels of NR2A, NR2B and NR2D mRNAs in the ANR1-treated striatal neurons was found after 24 h treatment (fig. 2B–D; table 1).

Effects of ANR1 Treatments on Expression of NR1 Proteins

Data were obtained from 18 separate Western blotting experiments. A reduction in NR1 protein was found in the striatal neurons treated with 5 μM ANR1 (fig. 3A). No change in the NR1 protein expression was found in the neurons treated with RNR1 or SNR1 (fig. 3A). Statistical analyses revealed that significant reductions (−51.85 ± 0.73%) were found in the striatal neurons treated with 5 and 20 μM of ANR1 (fig. 3B). Treatments with 100 μM of ANR1 also resulted in similar reduction of NR1 protein expression (data not shown).

Effects of ANR1 Treatments on Phosphorylation of NR1 Proteins

Data were obtained from fifteen separate Western blotting experiments. In the control experiments, bands of phosphorylated NR1 proteins were eliminated after treatment with phosphatase (fig. 4A). Similar to the above results, after treatment with 5 μM of ANR1, a significant reduction of NR1 proteins was seen (fig. 4B). However, levels of two phosphorylated forms of NR1 proteins, i.e. NR1 proteins phosphorylated at serine 896 and 897 residues, were not modulated after the ANR1 treatments (fig. 4B–E). Similar results were found after treatments with 20 and 100 μM of ANR1 (data not shown).

Effects of ANR1 Treatments on Patterns of NR1 Immunoreactivity

Data were obtained from 20 experiments. Double immunofluorescence revealed that most of the striatal neu-

Fig. 3. Western blotting analyses of the levels of NR1 proteins in striatal neurons treated with NR1 antisense oligos (ANR1), NR1 sense oligos (SNR1), NR1 random oligos (RNR1) and in control. **A** A single NR1-immunoreactive band of about 120 kDa is observed in each of the four groups. Reductions in the level of NR1 protein are observed only after treatment with ANR1 (5 and 20 μ M). Similar levels of NR1 protein are found in the SNR1 and RNR1 treatments and in the control. **B** Statistical analyses show that significant reductions in the expression of NR1 proteins are observed only after ANR1 treatments (5 and 20 μ M). Means $\pm$ SEM are shown. *** $p < 0.001$.

rons displayed immunoreactivity for GAD (fig. 5). They were therefore presumed to be GABAergic neurons. No difference in the patterns of distribution of NR1 immunoreactivity was observed after ANR1 treatments (fig. 5).

Effects of ANR1 Treatments on Physiology of NMDA Receptors

Data were obtained from recording of 36 striatal neurons. The neurons were observed by a DIC-infrared camera in the light microscope (fig. 6A). The neurons were injected with biocytin and were identified by fluorescence microscopy (fig. 6B). Subsequently, GABAergic striatal neurons were confirmed by the display of GAD immunoreactivity (fig. 6C).

In the GAD-positive striatal neurons with ANR1 treatments, NMDA-induced peak inward current was observed to be over 20 pA (fig. 7A, B). After 5 μ M ANR1 treatment, no statistical difference in the NMDA-induced peak inward currents was observed between the control

and the treatment neurons (fig. 7B). However, a significant reduction in NMDA-induced peak inward currents was observed in the striatal neurons treated with 20 or 100 μ M ANR1 (fig. 7B).

Fig. 4. Western blotting analyses of the levels of NR1 proteins and two phosphorylated forms of NR1 proteins at serine 896 (p NR1 s896) and 897 (p NR1 s897) residues in striatal neurons treated with NR1 antisense oligos (ANR1), NR1 sense oligos (SNR1), NR1 random oligos (RNR1) and in control. **A** Results of experiments before and after phosphatase pretreatments are shown. After phosphatase pre-treatments, bands of the phosphorylated NR1 proteins were abolished. **B** As shown in figure 2, a reduction of NR1 protein expression is observed only after ANR1 treatment (indicated by an arrow). No change in expressions of the two phosphorylated forms of NR1 proteins at serine 896 and 897 residues is found. **C–E** Statistical analyses reveal that a significant reduction is seen in total NR1 proteins. No significant change of the two phosphorylated forms of NR1 proteins is found. *** $p < 0.001$.

Fig. 5. Results of immunofluorescence analyses are shown. In glutamic acid decarboxylase (GAD)-immunoreactive striatal neurons (**A**, **C**, **E**), when compared with the control (**B**), NR1 antisense (ANR1) treatments with two concentrations (5 μ M in **D**; 20 μ M in **F**) result in no change in pattern of distribution of NR1 immunoreactivity. Scale bar (in **F**, for all micrographs) = 50 μ m. **Fig. 6.** **A** Differential interference contrast image of patched striatal neuron is shown. **B** The same neuron shown in **A** is revealed by the presence of biocytin (revealed by using avidin-rhodamine). **C** The same patched striatal neurons are then immunostained to reveal immunoreactivity for glutamic acid decarboxylase (GAD) to confirm the identity of GABAergic neurons in striatal culture. Scale bar (in **C**, for all micrographs) = 50 μ m.

Effects of ANR1 Treatments on NMDA-Induced Excitotoxic Cell Death

Data were obtained from 21 experiments. No observable cell death was found in striatal neurons treated with ANR1 (data not shown). Addition of 500 μ M of NMDA induced a high degree of cell death in the cultured striatal neurons (fig. 8A). A significant reduction of NMDA-induced cell death was observed when the cultures were treated with NMDA together with DL-APV (fig. 8B). High degree of cell death was observed in cultures co-treated with NMDA together with 5 μ M (fig. 8C) or 20 μ M (fig. 8D) of RNR1. Similarly, a high degree of cell death was also observed in cultures co-treated with NMDA and 5 μ M (fig. 8E) or 20 μ M (fig. 8F) of SNR1. In contrast, similar to DL-APV, neuroprotective effects were seen in cultures treated with NMDA together with 5 μ M (fig. 8G) and 20 μ M (fig. 8H) of ANR1. Statistical analyses revealed that significant reductions in NMDA-induced excitotoxicity were found only in co-treatments of NMDA with L-APV or 5 or 20 μ M of ANR1 (fig. 8I).

Discussion

The results of the present study indicate that ANR1 is a potent neuroprotective agent against NMDA-induced excitotoxic cell death in striatal neurons in vitro. The efficacy of ANR1 is also shown by the present results as one single dose of ANR1 is effective to reduce significantly both NR1 mRNA and protein expressions. The NMDA-induced inward currents are thus reduced by the application of ANR1. These findings as a whole indicate that ANR1 has potential therapeutic applications for neurodegenerative diseases.

ANR1 Is a Specific and Nontoxic Agent

The first important finding here is that ANR1 is specific for reducing the expressions of NR1 mRNA and protein. The present results have demonstrated the specificity of ANR1 as SNR1 and RNR1 results in no reduction of NR1 protein expression. There is also no change in expression of NR2 subunits after ANR1 treatments. These findings, however, do not preclude the possibility that ANR1 may have effects on other neurotransmitter systems.

Fig. 7. A Typical traces showing the whole-cell membrane current induced by 50 μ M NMDA in a striatal neuron. No change in current is observed after application of 5 μ M of NR1 antisense oligos (ANR1). In contrast, a reduction of the whole-cell membrane current is observed after the application of 100 μ M of ANR1. **B** Statistical analyses indicate that the whole-cell membrane currents induced by 50 μ M NMDA in the striatal neurons treated with 20 or 100 μ M of ANR1 are significantly reduced. * $p < 0.05$; ** $p < 0.01$.

Previous reports demonstrate that intraparenchymal applications of ANR1 in the neostriatum result in modulations in motor behaviors of rats [26, 29] and exert an amelioration of motor symptoms in parkinsonian rats

[28]. Compared to the previous in vivo studies [26, 27, 29], an equivalent amount of ANR1 has been applied to striatal neurons in culture. The present results thus extend our previous in vivo observations [26, 28] that ANR1

Fig. 8. Neuroprotective effects of NR1 antisense oligos (ANR1) are shown. In primary cell culture, there is only a low number of striatal neurons which can survive after the treatment of 500 μ M of NMDA (**A**). The number of survived striatal neurons is seen to increase after co-treatment using 500 μ M of NMDA with 1 mM of DL-APV (**B**). Co-treatments of NMDA with NR1 random oligos (RNR1: 5 or 20 μ M, **C–D**) or NR1 sense oligos (SNR1: 5 or 20 μ M, **E–F**) do not increase the number of survived striatal neurons. In contrast, the numbers of survived striatal neurons are seen to increase after co-treatments of NMDA with ANR1 (5 or 20 μ M, **G–H**). Statistical analyses reveal that significant reductions in NMDA-induced toxicity are only found in striatal neurons treated with ANR1 (5 and 20 μ M) and DL-APV (**I**). * $p < 0.05$.

is capable to reduce the amount of NR1 mRNAs, and importantly proteins in cultured striatal neurons up to the extent of around 30% reduction. ANR1 is found to exert its actions directly on striatal neurons and is thus capable of inducing robust modulations in motor behaviors.

The second important finding is that ANR1 is non-toxic to striatal neurons as shown by the present NMDA toxicity assays and patch clamp analysis. Toxic effects of ANR1, if any, are far more difficult to observe in previous *in vivo* studies [26, 28]. The present results thus demonstrate that application of ANR1 up to 100 μM do not induce neuronal cell death.

In addition, immunofluorescence also demonstrated that there is no change in the pattern of distribution of NR1 immunoreactivity. This observation reflects that the aftermath effect of ANR1 does not modulate intracellular distribution of the NR1 proteins as there is less effect of ANR1 in the phosphorylation of NR1 at serine residues (see below), in which phosphorylation of NR1 is related to the subcellular distribution of NMDA receptors [40–42]. ANR1 may therefore affect the dynamic metabolism of NR1 protein synthesis.

ANR1 Directly Modulates NMDA Receptor Physiology

NR1 is the principal subunit of a functional NMDA receptor [10, 12, 14]. NMDA channels are mixed ion channel [15, 43, 44]. Two major ions that can go through the NMDA channels are Na^+ and Ca^{2+} ions [15, 43, 44].

A reduction of NMDA-mediated inward current in general reflects a reduction in Na^+ influx in the striatal neurons through the NMDA channels. These observations have raised an interesting point. The present RT-PCR and Western blot analyses have revealed that different concentrations of ANR1 employed (low concentration: 5 μM ; high concentration: 20–100 μM) have similar potency in reduction of NR1 mRNA and protein expressions. The effects of different concentrations of ANR1 on the NMDA channel activities, in theory, should remain the same. Interestingly, it seems to be not the case in Na^+ influx through the NMDA channel as indicated by whole-cell patch clamp analysis. The present results thus suggest Na^+ influx through the NMDA channels is less sensitive than to a reduction of NR1 protein synthesis. This is very different to the issue of Ca^{2+} influx as excitotoxic cell death is reduced by both 5 and 20 μM of ANR1 (see below). This implies an application of low concentration of ANR1 may exert a lower degree of impairment as in the case of higher concentrations of ANR1 in terms of NMDA receptor-mediated peak inward currents despite low and

high concentrations are capable to reduce the gene expressions of NR1.

Different concentrations of ANR1 may affect the turnover of different pools of NR1 proteins that may in turn differentially affect the receptor physiology. Although this explanation remains hypothetical, previous studies [45, 46] have indicated that there are at least two degrading pools of NR1 proteins, i.e. a fast and a slow degrading pool, found in cerebellar granule cells or in PC12 cells. A low concentration of ANR1 is likely to be degraded quickly within the cytosol. In the case where higher concentrations of ANR1 are applied, a longer retention time of ANR1 within the neurons is expected. The fast degrading NR1 proteins demonstrated a half-life of 1.6 h and this pool of NR1 proteins is most likely to be affected by both low and high concentrations of ANR1. Previous studies have demonstrated that intracerebral injections of antisense signals can be detected in the brain 16 h after the application [47, 48]. It is anticipated that the retention time of ANR1 inside neurons in culture may be even longer. The slow degrading pool of NR1 proteins were found to have a half-life of 16.1 h [45, 46] and this pool of NR1 may be less likely to be affected by 5 μM of ANR1. The two pools of NR1 proteins are suggested to be composed of different splice variants, i.e. NR1–4a in fast degrading pool and NR1–2a in slow degrading pool, respectively [46]. There is no precise information about the functional roles of the two pools of NR1 proteins, e.g. their roles in Na^+ conductance in the NMDA receptor complex. It is also unknown whether the ANR1 has differential effects on the synthesis of the two splice variants of the NR1 protein. The present data thus suggest that at least there is such a possibility. The functional roles of different pools of NR1 proteins remain to be established. One useful tool in the future may be a splice variant specific ANR1.

In addition, the disparities between gene expressions and NMDA receptor physiology could be explained by the fact that RT-PCR, Western blotting and patch clamp analysis are different techniques with different detection sensitivities. A recent study shows that application of NR1 antisense oligos (a different sequence) to the rat spinal cord results in a 30% reduction of NR1 mRNA in the dorsal horn, whereas a 50% reduction in NMDA receptor ligand binding is found [49]. These results are consistent with our present observation in striatal neurons and show that the effects of NR1 antisense can exert different degrees of impairment on NR1 gene expressions and on NMDA receptor functions.

Effects ANR1 on Phosphorylation of NR1 at Serine Residues

One straight observation of the present results demonstrates that ANR1 does not modulate the amount of phosphorylated NR1 proteins at two serine residues, ser896 and ser897. The present results, however, do not preclude the possibility that there is an increased level of phosphorylated NR1 proteins because there is an overall reduction of NR1 proteins after the antisense treatment. This increase in the phosphorylation level may be a mechanism that compensates the reduction of NR1 expression level. Despite the above uncertainty whether there is no change or an increase of total phosphorylated NR1 proteins, the present results are capable to demonstrate that the amounts of phosphorylated NR1 proteins are not reduced after antisense treatment.

Phosphorylation of the NR1 proteins is an important regulation of NMDA receptor functions [50, 51]. Phosphorylation of NR1 proteins affect the anchorage of the NMDA receptor to plasma membranes and the subcellular distribution of the receptor [52–54]. Interestingly, in parkinsonian animal models, conflicting results in terms of modulation of phosphorylated NR proteins have been described. Dunah et al. [55] have found a decrease of NR1 phosphorylation at ser890 and ser896 but no change in NR1 phosphorylation at ser897. However, subsequent study shows that a significant increases of phosphorylated NR1 proteins at ser897 has been found in the neostriatum of the rat model [56]. A recent report shows that ANR1 has been shown to ameliorate the parkinsonian motor symptoms in a rat parkinsonian model [28]. The present results directly demonstrate that ANR1 does not reduce the phosphorylation of NR1 proteins at these two serine residues. These results show that the beneficial effects of ANR1 in the parkinsonian rat model may not act upon the phosphorylation of NR1 protein. The precise roles of NR1 phosphorylation in the parkinsonian conditions remain to be established.

ANR1 Is an Effective Neuroprotective Agent

Another novel finding of the present study is the neuroprotective effects of ANR1 on the striatal neurons against NMDA-induced excitotoxicity. Excitotoxic cell death is known to be mediated by influx of Ca^{2+} into neurons [57, 58]. The present results indicate that the both 5 and 20 μM of ANR1 are similar in terms of potency for neuroprotection. As discussed, both concentrations of ANR1 can reduce NR1 protein expression to a similar degree. This reduction in NR1 protein expression is presumed to reduce the number of Ca^{2+} -permeable NMDA

receptors in the neurons. Interestingly, the degree of neuroprotection against NMDA toxicity of ANR1 is similar to L-APV, a well-known competitive NMDA receptor antagonist that has neuroprotective potency [59]. These findings indicate that ANR1 may be developed in the future as a neuroprotective agent against excitotoxic cell death in motor disorders and other neurodegenerative diseases.

In summary, a single dose of ANR1 is effective in reduction of the gene expression of NR1 proteins in striatal neurons in culture. ANR1 is non-toxic and specific and this NR1-specific oligo directly modulates the NMDA channel activity and thus offers neuroprotection against NMDA-induced excitotoxicity in striatal neurons. Therefore, ANR1 may be a potential therapeutic agent in gene therapy and have implications in treatment of neurodegenerative diseases.

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